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14. ABSTRACT

Aberrant regulation of the epidermal growth factor receptor is implicated in a number of epithelial cancers, including a large fraction of breast cancers. Antibody based inhibitors directed against the extracellular ligand binding domain of the receptor have proven to be a clinically viable cancer therapeutic. We have used mutagenesis and binding competition studies to map the epitopes of two inhibitory antibodies. Additionally, we have structurally and biochemically characterized a set of inhibitory single-domain VHH antibody fragments directed against the exctracellular domain of the receptor.

15. SUBJECT TERMS
EGFR, antibody, inhibitor

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Introduction

Breast cancer is a leading cause of cancer-related death worldwide, and has a particularly high incidence in the United States. The occurrence of breast cancer is correlated with hereditary and environmental factors, and its prognosis is linked to a large number of proteins and cellular pathways. One such protein is the epidermal growth factor receptor (EGFR/ErbB1/HER1), a plasma membrane receptor tyrosine kinase. Activation of EGFR promotes cell growth, proliferation, and differentiation. Aberrant regulation of EGFR activation is associated with tumor growth, malignancy, and poor prognosis. EGFR expression is observed in a number of epithelial cancers, including those of the colon, pancreas, lung, head and neck, and up to 80% of breast cancers [1]. Strategies to therapeutically inhibit activation of EGFR target either the intracellular tyrosine kinase domain, or the extracellular ligand binding region. Examples of both approaches have received FDA approval [2, 3].

We have previously structurally characterized three monoclonal antibody (mAb) inhibitors that target the EGFR extracellular region: cetuximab/mAb C225 (Erbitux; ImClone) [4], mAb IMC-11F8 (ImClone) [5], and matuzumab/mAb EMD72000 (Merck KGaA) [6]. These studies have yielded valuable insight into the mechanisms of receptor activation and antibody inhibition.

While a number of antibody based inhibitors exist and are under investigation, there remains interest in identifying new inhibitors that may exhibit different receptor binding properties, present fewer clinical side effects, or be amenable to antibody combination therapies. In this study we apply existing structural knowledge to the inhibitory monoclonal antibodies 108 and 13A9, which are noted for their unique inhibitory effects against EGFR [7, 8]. In addition, we structurally characterize a panel of camelid derived single domain inhibitory VHH antibody fragments [9].

[Please note that the original Statement of Work (SOW) details a research plan pertaining to a different project. The original research plan focused on inflammation, innate immunity, and the inflammasome protein complex. However, in 2007 a competing group published a body of work that substantially overlapped with the research plan put forth in the original SOW. A revised SOW covering the research topic introduced above was submitted to and approved by the funding agency. A copy of the approved revised SOW is included in the Appendix.]

Body

Original Statement of Work

As described in prior progress reports, expression of the various components of the inflammasome complex was attempted in *E. coli* and baculovirus/Sf9 insect cell expression systems, with limited success. The multi-domain protein NALP, its individual domains (pyrin, NACHT, and LRR), and inflammasome adaptor proteins Cardinal and ASC were cloned. Multiple tagging strategies, including 6xHis, GST, and intein/chitin binding domains were attempted for purification. The NALP2 pyrin domain was found to express well in bacteria, as was the adaptor protein, Cardinal. However, the Cardinal suffers from rapid proteolysis over the course of the purification procedure. Limited amounts of full-length NALP2 were produced in Sf9 cells, and this protein found to be proteolytically stable.

The NALP2 pyrin domain formed crystals (Fig. 1), but could not be refined to sufficient size and quality for data collection.

Full-length NALP2 was analyzed by analytical size exclusion chromatography and found to exist in apparent monomer-oligomer equilibrium (Fig. 2).

Revised Statement of Work

Task 1. Produce and purify proteins. (months 18-36)

Monoclonal antibodies were acquired as pure protein through existing collaborations. These antibodies include mAb C225 (i.e., cetuximab; ImClone), mAb 425 (i.e., the murine version of matuzumab; Genentech), mAb 108 (J. Schlessinger, Yale), and mAb 13A9 (Genentech). Antibody C225 and the humanized version of 425 (EMD72000) have previously been structurally characterized by the Ferguson lab [4, 6]. Antibodies 108 and 13A9 have not been structurally characterized, but exhibit unique properties as EGFR inhibitors. mAb 108 specifically blocks ligand binding to the high affinity population of receptors on the cell surface [7]. mAb 13A9 blocks binding of the ligand TGFα, but not EGF [8] .

Fab fragments were generated from these antibodies by papain cleavage and were separated from Fc fragments and uncleaved antibody by protein-A affinity chromatography (Fab Preparation Kit, Pierce). Fabs were further purified by size exclusion chromatography (SEC250, BioRad). Purified Fabs were spin concentrated (Vivaspin, Sartorius) to a concentration between 5 and 10 mg/ml in 10mM HEPES pH 8.0, 150mM NaCl.

A panel of anti-EGFR llama VHH antibody fragments, designated 7D12, IA1, 9G8, and B4 (R. Roovers, Utrecht University), have also been purified. DNA encoding these proteins was cloned into expression plasmid pET-22b (Novagen), resulting in periplasmically targeted C-terminally his-tagged constructs. VHHs were overexpressed in Rosetta2(DE3) *E. coli* by 4h IPTG induction. Bacteria were pelleted and resuspended in PBS, and VHHs were liberated from the periplasmic space by freeze-thaw. VHH constructs were purified from periplasmic isolates by Ni-NTA (Qiagen) affinity chromatography, followed by size exclusion chromatography (Superose12, GE Healthcare). Purified VHHs were spin concentrated to a concentration of approximately 10 mg/ml and stored in 10mM HEPES pH 8.0, 150mM NaCl.

Constructs comprising portions of the extracellular region of the EGFR were generated as secreted protein by baculovirus infection of Sf9 cells, as described [4, 6]. Soluble EGFR ectodomain (sEGFR) was produced and purified, as were shorter constructs, including EGFR domain 3 alone (sEGFRd3) [5].

Task 2. Assay interaction between antibody inhibitors and sEGFR (months 20-30)

The binding of sEGFR to immobilized Fab fragments was assayed by surface plasmon resonance studies (SPR, Biacore). All SPR studies were conducted in 10mM HEPES pH 8.0, 150mM NaCl, 3mM EDTA, 0.0005% Tween20. Dissociation constants were determined by measuring the equilibrium binding response of free sEGFR to immobilized Fab, over a range of concentrations of sEGFR. Data were fit by nonlinear regression to a Langmuir binding isotherm (Table 1).

	Fab C225	Fab 425	Fab 108	Fab 13A9	EGF
Kd	2.3 ± 0.3 nM	46 ±.4 8.7 nM	19.8 ± 2.2 nM	1.1 ± 0.1 nM	130 ± 4 nM

Table 1. Measured dissociation constants for the interaction between inhibitory Fabs or ligand and sEGFR. Fab or ligand was immobilized on an SPR chip by amine coupling. Varying concentrations of free sEGFR was flowed over this surface, and the equilibrium binding response was measured at each concentration.

Binding constants for VHH constructs were assayed by analogous SPR studies, with immobilized VHH and free sEGFR. Approximate dissociation constants have been determined, with values on the order of 100 uM. This is approximately 10 to 100 fold weaker than observed for Fabs, and is reasonable given the smaller paratope surface of VHHs compared to Fabs. However, immobilized VHHs were found not to tolerate surface regeneration conditions required for collection of replicate data. Consequently, we are currently attempting alternative approaches to accurately assay the binding affinities of the VHHs.

As a preliminary experiment to crystallographic studies (Task 5), VHHs 7D12 and IA1 were analyzed alone and in complex with receptor by sedimentation velocity analytical ultracentrifugation. Ultracentrifugation studies were conducted in a Beckman XL-A analytical ultracentrifuge, at rotor speeds of 42500 to 45000 rpm. Hydrodynamic parameters of proteins and solution were estimated with the

software SEDNTERP [10], and data analysis was performed with the program Sedfit [11]. VHH 7D12 and sEGFRd3 were observed to form a 1:1 stoichiometric complex (Fig. 6), revealing that the 7D12 epitope lies on domain 3. VHH IA1 and sEGFR were found to bind with 1:1 stoichiometry (Fig. 5). IA1 was also observed to bind simultaneously with Fab C225, suggesting non-overlapping binding sites.

Task 3. Assay ability of inhibitor to compete with ligand/sEGFR binding (months 24-36)

Initial characterization of the competition of Fab for ligand binding to sEGFR was accomplished using SPR. A saturating concentration of sEGFR (600 nM), along with a 10-fold molar excess of Fab or EGF ligand, was passed over a surface of immobilized EGF. The resulting binding response was measured and compared to the response in the absence of Fab or free ligand (Fig. 7). All Fabs were observed to inhibit sEGFR binding to EGF to some degree. Fab C225 essentially abolishes ligand binding, which corresponds well with its structurally characterized function as a high-affinity competitive inhibitor [4]. Fab 425 only partially blocks binding to EGF, even though the Fab 425/sEGFR interaction is three-fold tighter than the binding of EGF to sEGFR. This partial decrease in ligand binding is consistent with conformational effects, rather than direct steric inhibition, which is supported by published structural characterization of the interaction [6]. Fabs 108 and 13A9 also exhibit weak inhibition of ligand binding, also suggesting conformational effects.

To compare the binding properties of the structurally characterized Fabs to those for which no structural data exists, cross-competition SPR binding studies were conducted. The binding of 100nM sEGFR to immobilized Fab was monitored in the presence of a 10-fold excess of the same or a different Fab. These data indicate that the Fabs in this panel possess distinct but overlapping epitopes on domain 3 of EGFR.

Task 4. Characterize binding footprints of inhibitory antibodies (months 24-30)

We have created a panel of sEGFR variants with point mutations on domain 3 that affect binding of EGF, Fab C225, or Fab 425, based on prior structural studies [4, 6]. The dissociation constants for the interactions of each variant with each inhibitory Fab have been determined by SPR. These data, represented as fold change in Kd, have been plotted on the surface of domain III (Fig. 4). This limited fine epitope map provides information on the location of the Fab 108 and 13A9 epitopes, in the absence of direct structural data.

We have further generated a panel of VHH variants, based on new structural data of VHHs bound to sEGFR (see Task 5). These variants incorporate point mutations in the VHH paratope, selected to weaken or strengthen the interaction of the VHHs with sEGFR. The dissociation constants of these VHH variants will be determined once a new binding assay has been optimized (see Task 2).

Task 5. Structurally characterize llama V_{HH} inhibitors by X-ray crystallography (months 20-36)

As described in the previous progress report, we have determined the X-ray crystal structure of the inhibitory VHH IA1 alone, to 1.55 Å. In addition, we have now determined X-ray crystal structures of three inhibitory VHHs in complex with sEGFR.

VHH 7D12 was purified in complex with sEGFRd3 by size exclusion chromatography and crystallized by hanging drop vapor diffusion. Crystals formed in 22.5% PEG3350, 12.5% glycerol, 0.1 M KI, 0.1M MES, and diffracted to 3.0Å. Data were processed with the software packages HKL-2000 [12] and CCP4 [13]. Molecular replacement was accomplished with the program Phaser [14]. Model building was done with Coot [15], and refinement with Refmac [16]. 7D12 appears to be a competitive inhibitor for ligand binding to EGF. Its epitope extensively overlaps with that of EGF, and partially overlaps with that of Fab C225 (Fig. 8).

VHH IA1 was purified in complex with sEGFR by size exclusion chromatography, but the resulting complex did not form crystals in any of the screened conditions. It is known that the extracellular portion

of EGFR does not crystallize on its own. This is likely due to its extensive glycosylation, and thus insufficient surface area for the formation of stable crystallographic contacts. A single bound VHH domain may not provide enough additional surface for stable crystal formation.

We hypothesized that the addition of bound Fab would provide sufficient additional protein surface area to provide crystal contacts. To this end, we purified a tertiary complex consisting of sEGFR, IA1, and Fab C225, by size exclusion chromatography. This complex formed crystals by hanging drop vapor diffusion in 22.5% PEG3350, 1.25M NaCl, 10% glycerol, 0.1M MES pH 6.5. Diffraction data was collected to 3.05Å. In the resulting refined model, IA1 binds a novel epitope on domain 3, adjacent to the C-terminal region of domain 2 (Fig. 9). In this complex, EGFR adopts a “tethered” conformation similar to that observed in the low pH and Fab C225-bound structures [4, 17], but with a slight rotation in the orientation domains 1 and 2 to domains 3 and 4, and possible disruption of the tether interactions.

The same strategy was utilized to obtain crystals of a VHH 9G8/Fab C225/sEGFR complex, which yielded diffraction data to 2.8Å. Model refinement of the 9G8 complex is not yet complete, but the 9G8 epitope appears to be almost identical to that of IA1, in spite of significant differences in the primary sequences of the two VHHs.

Key research accomplishments

Inflammasome characterization (Original SOW):

- Multi-microgram amounts of full-length NALP2 have been produced in Sf9 cells and purified to homogeneity.
- The NALP pyrin domain has been purified from *E. coli,* and initial crystals of this domain have been grown.
- Full-length NALP has been assessed to be in apparently monomer/oligomer equilibrium by size exclusion chromatography.

Interactions of Epidermal Growth Factor Receptor with inhibitory antibodies (revised SOW):

- A panel of inhibitory Fab and VHH fragments has been purified and can be used for subsequent biochemical experiments.
- Affinity constants for the binding of Fab fragments to sEGFR have been determined, as have initial values for the binding of VHHs to sEGFR.
- Inhibitory Fab fragments have been shown to compete with EGF ligand for binding to sEGFR.
- Cross-competition and fine epitope mapping studies have provided approximate epitopes for inhibitory Fabs 108 and 13A9.
- The crystal structure of one VHH construct, IA1, has been determined in the unbound state.
- The crystal structures of three VHH constructs (7D12, IA1, 9G8) bound to portions of EGFR extracellular domain have been determined.

Training

- The PI has acquired proficiency in standard cloning methods, protein expression techniques in *E. coli* and Sf9 cells, and protein purification strategies.
- The PI has attended the Analytical Ultracentrifugation Workshop at the National Institutes of Health, funded by The Foundation for Advanced Education in the Sciences. This workshop focused on experimental design and data interpretation for hydrodynamic and thermodynamic techniques, including sedimentation equilibrium and sedimentation velocity analytical ultracentrifugation, dynamic light scattering, and isothermal titration calorimetry. These techniques all have strong relevance to the PI's field of study and to the aims of the project funded by this grant.
- The PI has attended the National School on Neutron and X-ray Scattering, held jointly at Argonne National Laboratory and Oak Ridge National Laboratory. This two week course offered intensive training on scattering techniques, taught by experts in the field. Neutron and X-ray scattering methods have application as biophysical tools for the characterization of biological molecules, and compliment higher resolution structural studies in the characterization of proteins and protein interactions.
- The PI has acquired extensive experience in X-ray crystallography techniques, including instrument operation, data collection, and data processing with in-house X-ray generators/diffractometers, as well as high flux X-ray beamlines at synchrotron radiation facilities. This experience includes extensive hands-on training on over 20 data collection trips with the Ferguson laboratory to beamlines at MacCHESS (Macromolecular Diffraction Facility at the Cornell High Energy Synchrotron Source), NSLS (National Synchrotron Light Source), and APS (Advanced Photon Source).
- The PI has gained experience with SAXS (small angle X-ray scattering) from several trips with the Ferguson lab to MacCHESS.
- The PI has achieved proficiency with the algorithms and software used for crystallography and SAXS data collection and processing. This includes determination of X-ray crystal substructure

by molecular replacement and experimental phasing methods, refinement of crystallographic models, and molecular envelope calculation from X-ray scattering data.

- The PI was the recipient of a Travel Award by the American Society for Biochemistry and Molecular Biology to attend the Experimental Biology 2009 meeting, where the PI presented a poster and talk. While at the meeting, the PI attended career development and networking events held for travel awardees.

Reportable Outcomes / Bibliography

The research supported by this award will serve as the basis for a portion of the PIs PhD thesis, which the PI expects to defend in Fall 2009.

Presentations:

June 2008: DOD Breast Cancer Research Program Era of Hope Meeting, "Binding Specificity of Inhibitory Antibodies to the Extracellular Region of Epidermal Growth Factor Receptor."

Publications:

Schmitz K.R., K.M. Ferguson. VHH antibodies as inhibitory molecules against the EGFR ectodomain. *In preparation.*

Schmitz K.R., S. Li, Y-S. Huoh, K.M. Ferguson. Specificity in interaction of inhibitory antibodies with the extracellular region of the EGF Receptor. *In preparation.*

Schmitz K.R., K.M. Ferguson. Interaction of antibodies with ErbB receptor extracellular regions. Exp Cell Res. 2009 315(4):659-70.

Schmitz K.R. ,K.M. Ferugson, Binding specificity of inhibitory antibodies to the extracellular region of epidermal growth factor receptor. Era of Hope Department of Defense Breast Cancer Research Program Meeting Proceedings. 2008: 390. *Abstract*.

Conclusion

Aberrant activation of the epidermal growth factor receptor (EGFR) is associated with a number of epithelial derived cancers, including up to 80% of breast cancers [1]. EGFR has been shown to be a viable clinical target, and a number of therapeutics directed against this receptor are under investigation or have already received FDA approval [2, 3]. A subset of these therapeutics includes antibodies against the extracellular ligand binding region of EGFR. Biochemical and structural studies of inhibitory EGFR antibodies may elucidate the mechanisms by which they function, and yield insight into the details of receptor activation.

Our work leverages existing structural data on well-characterized anti-EGFR antibodies (such as cetuximab and matuzumab [4, 6]) to better understand the inhibitory properties of antibodies for which no structural information exists. In particular we have identified approximate epitopes for antibodies 13A9 and 108. These inhibitory antibodies are notable for their unusual inhibitory properties. By understanding how the binding of different antibodies leads to receptor inhibition, we hope to guide development of new and better inhibitors, and improved clinical efficacy through combination therapies.

Additionally, we have structurally and biochemically characterized three novel inhibitory molecules belonging to a distinct class of antibodies – camelid VHH fragments. We show that these inhibitors bind to novel epitopes on EGFR, but inhibit activation by mechanisms similar to those of monoclonal IgG antibodies. VHH inhibitory antibodies are smaller, more easily produced and engineered, and potentially more versatile than traditional monoclonal inhibitory antibodies [9]. This research may ultimately lead to rationally designed EGFR inhibitors for immunohistological or therapeutic applications.

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Appendix

Statement of Work

Task 1. Produce and purify proteins. (months 18-36)

- Acquire partially purified monoclonal IgG antibodies through existing collaborations (months 18-24)
- Cleave IgG and isolate antibody F_{ab} fragments (months 18-24)
- Acquire genes for llama V_{HH} antibody fragments through existing collaboration (months 18-22)
- Clone llama V_{HH} fragments, optimize bacterial expression and purification strategy (months 20-24)
- Produce and purify panel of llama V_{HH} antibody fragments (months 20-36)
- Produce and purify sEGFR, single-domain sEGFR constructs, and variants (months 20-36)

Task 2. Assay interaction between antibody inhibitors and sEGFR (months 20-30)

- Surface plasmon resonance studies between F_{ab} and sEGFR (months 20-25)
- Surface plasmon resonance studies between V_{HH} and sEGFR (months 25-30)
- Sedimentation equilibrium analytical ultracentrifugation studies (months 25-30)

Task 3. Assay ability of inhibitor to compete with ligand/sEGFR binding (months 24-36)

- Competition between antibody inhibitors and ligand (months 24-30)
- Competition between individual antibody inhibitors (months 30-36)

Task 4. Characterize binding footprints of inhibitory antibodies (months 20-30)

- Screen inhibitors against panel of sEGFR surface residue mutants (months 20-30)
- Design and generate new sEGFR point mutations based on crystallographic data (months 24-30)
- Screen inhibitors against second panel of sEGFR surface mutants (months 30-36)

Task 5. Structurally characterize llama V_{HH} inhibitors by X-ray crystallography (months 20-36)

- Identify crystallization conditions for V_{HH} inhibitors (months 20-24)
- Identify crystallization conditions for inhibitor/sEGFR complexes (months 24-30)
- Test crystals for diffraction (months 20-30)
- Collect high-resolution diffraction data (months 24-36)
- Build and refine models of inhibitors and complexes (months 24-36)
- Analyze crystal structures (months 24-36)
- Characterize conformation of inhibitor/sEGFR complexes by DLS and SAXS (months 24-30)

Supporting Data

Figure 1. Purified NALP2 pyrin domain forms crystals in 50% 2-methyl-2,4-pentanediol, 0.2 M ammonium acetate, 50 mM acetate pH 5.0.

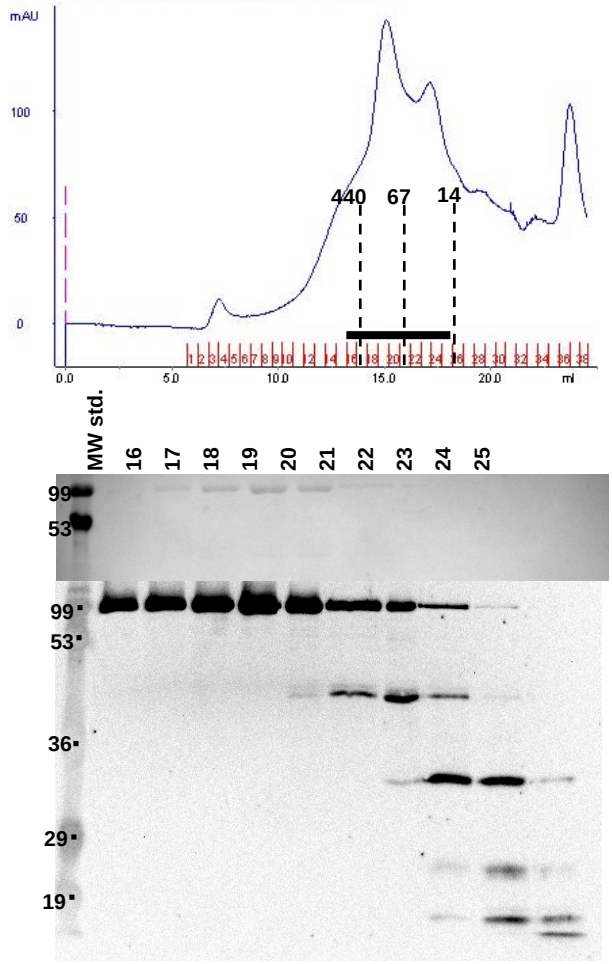
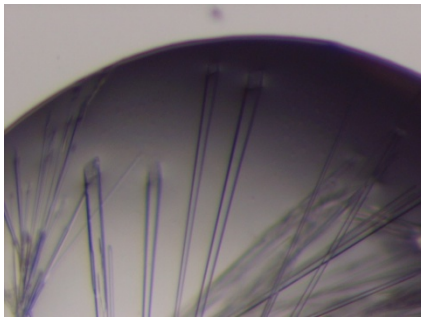


Figure 2. Full-length NALP2 can be purified to homogeneity from Sf9 cells, and exists in a monomer-oligomer equilibrium. (A) Chromatogram of NALP2, expressed in Sf9 cells, elution profile from a Superose 6 size exclusion column. Black line denotes fractions that were further analyzed by SDS-PAGE. Elution volumes of size standards are noted (kDa). (B) Ponceau (upper) and anti-tetraHis immunoblot (lower) showing elution fractions from chromatogram in A. NALP2 is present at low concentration, but is separated from its degradation products. A peak and a shorter elution volume shoulder correspond to elution of full-length NALP2, suggesting monomer-oligomer equilibrium.

A

Space group: $P4_3$	**IA1**
Resolution	1.55 Å
Completeness	99.2 (94.5)
I/sigma	47.5 (4.4)
R_{sym}	0.037 (0.283)
Bond Length RMS	0.007
Bond Angle RMS	1.138
$R_{working}$ (R_{free})	0.22 (0.25)

B



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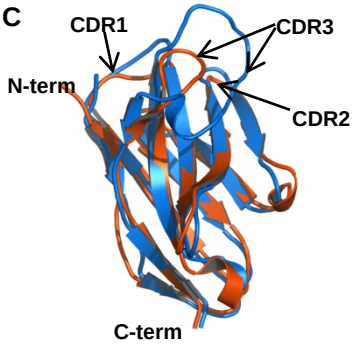

Figure 3. Crystal structure of IA1 llama V_{HH} antibody. The structure of antibody IA1 was determined to a resolution of 1.55 Å. Data were collected at the Advanced Photon Source, beamline 23 ID-B. Data collection and refinement statistics (for the current stage of refinement) are given in A. Values in parenthesis reflect the highest resolution shell of data. RMS = root mean square deviation. (B) Rod-like crystals (75x75x500 um) formed in 30% PEG8000, 0.2M ammonium sulfate, 0.1 M MES, pH 6.0. (C) Cartoon representation of IA1 (blue) overlaid with the N-terminal Ig domain of the C225 heavy chain (from PDB 1yy8). Differences between the two structures reside chiefly in the complementarity determining region (CDR) loops. Particularly, CDR3 is much longer in IA1 and folds against the surface of the Ig domain.

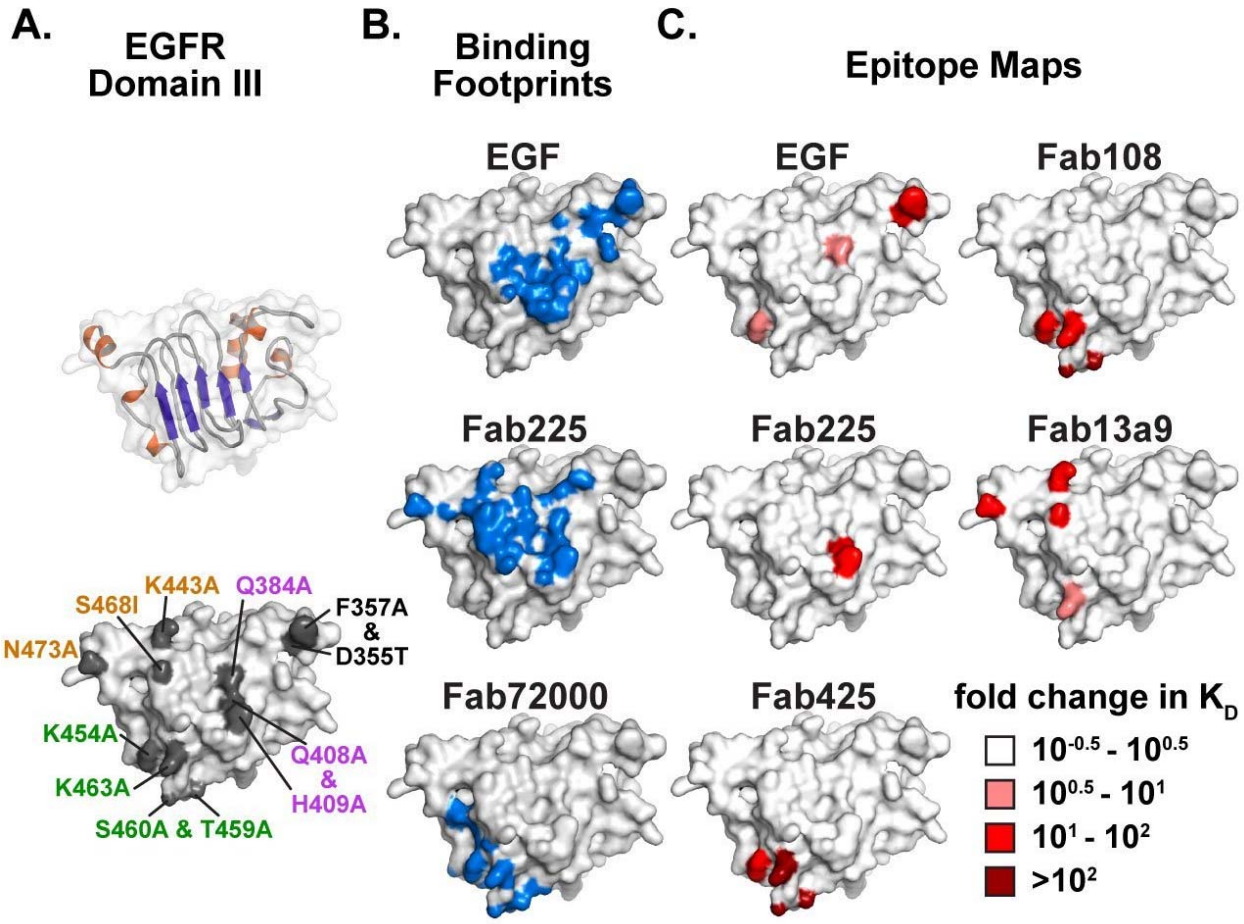

Figure 4. Limited fine epitope mapping of Fab epitopes on domain 3 of EGFR. A) Cartoon representation of domain 3, and a surface representation of residues altered (gray) in the panel of point mutants. Residues labeled in black affect binding of EGF; those in gold affect Fab C225 binding; those in pink affect both EGF and Fab C225; those in green affect FabEMD72000 (and its murine analog, Fab 425). B) EGF binding site and Fab epitopes (blue) shown on surface representations of domain 3 (generated from pdbs 1IVO, 1YY9, and 3C09). C) Fold change in Kd of the indicated Fab against each point mutant, represented by residue color, as indicated.

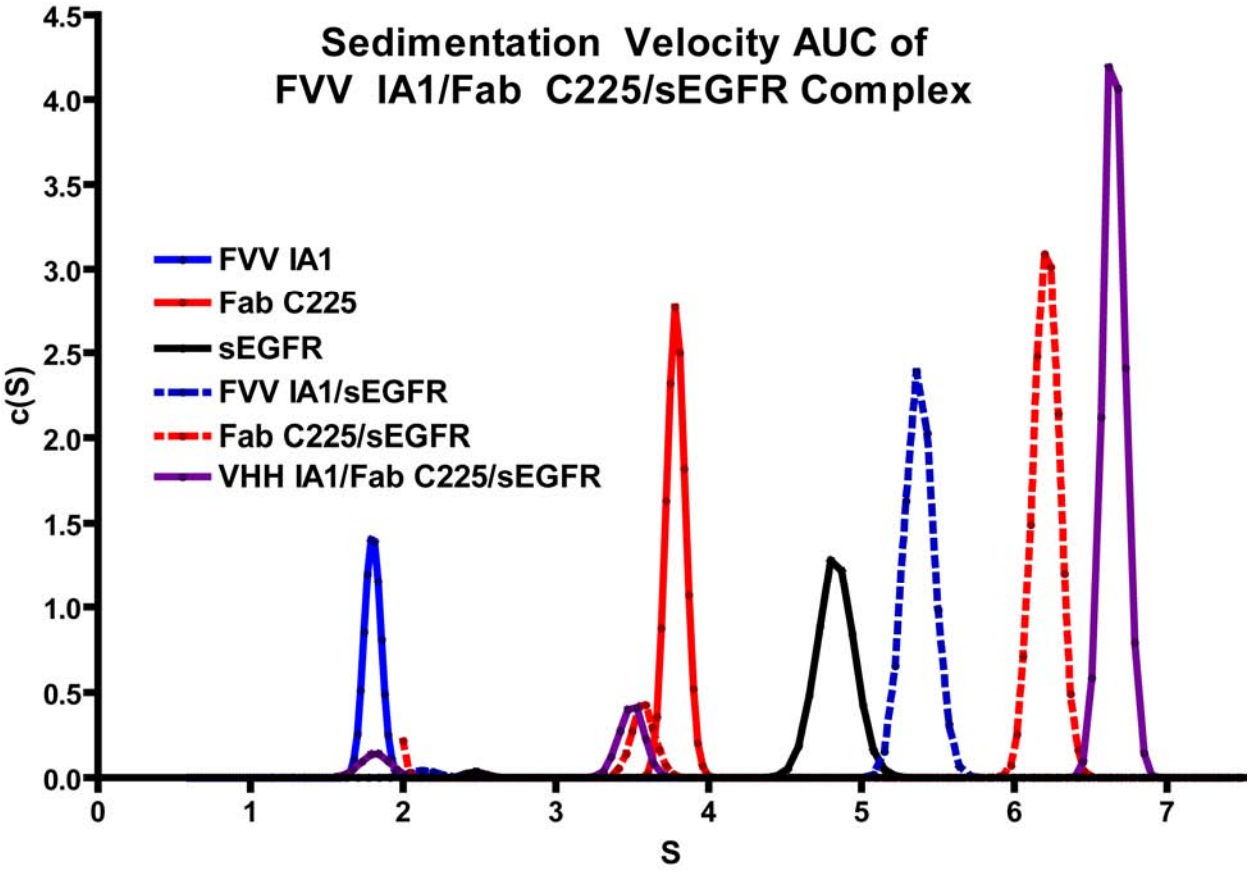

Figure 5. VHH IA1 and Fab C225 bind simultaneously to sEGFR. Sedimentation velocity analytical ultracentrifugation species distribution c(S) analysis of 5uM VHH IA1 alone (solid blue), 5uM Fab C225 alone (solid red), 5uM sEGFR alone (black), 5uM VHH IA1 with 5uM sEGFR (dashed blue), 5uM Fab C225 with 5uM sEGFR (dashed red), and 5uM VHH IA1 with 5uM Fab C225 and 5uM sEGFR (violet).

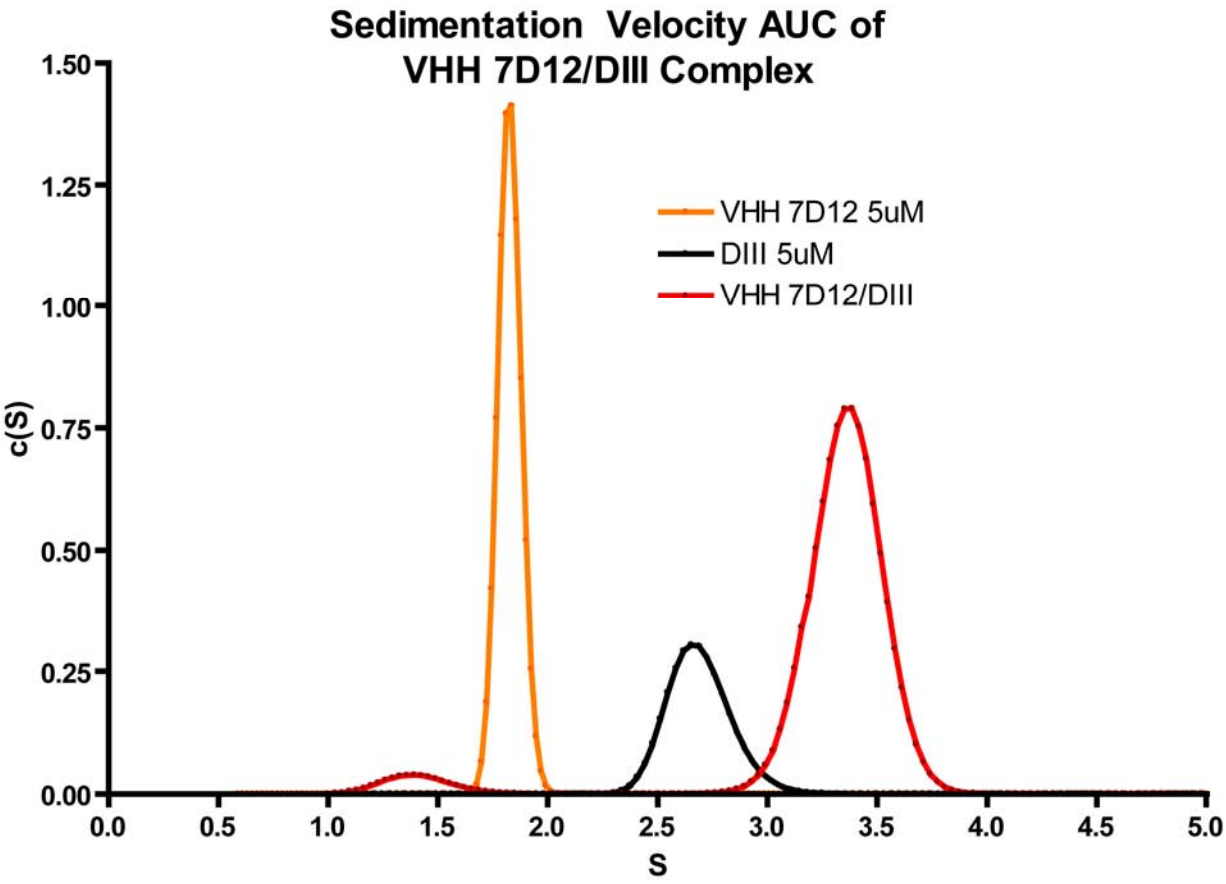

Figure 6. VHH 7D12 binds domain 3 of EGFR. Sedimentation velocity analytical ultracentrifugation c(S) species distribution analysis of 5µM VHH 7D12 alone (orange), 5µM sEGFRd3 alone (black), and 5µM VHH 7D12 and 5µM sEGFRd3 (red). VHH 7D12 recognizes an epitope on domain 3 of EGFR.

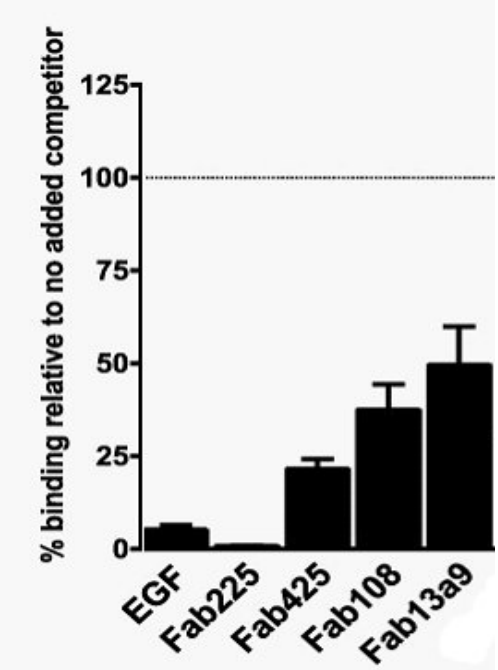

Figure 7. Inhibitory Fabs compete with EGF ligand for binding to sEGFR. 600 nM of sEGFR, plus a 10-fold excess of the indicated Fab, or EGF, was passed over a surface of immobilized EGF. Residual binding is shown as a % of equilibrium binding in the absence of free Fab or EGF.

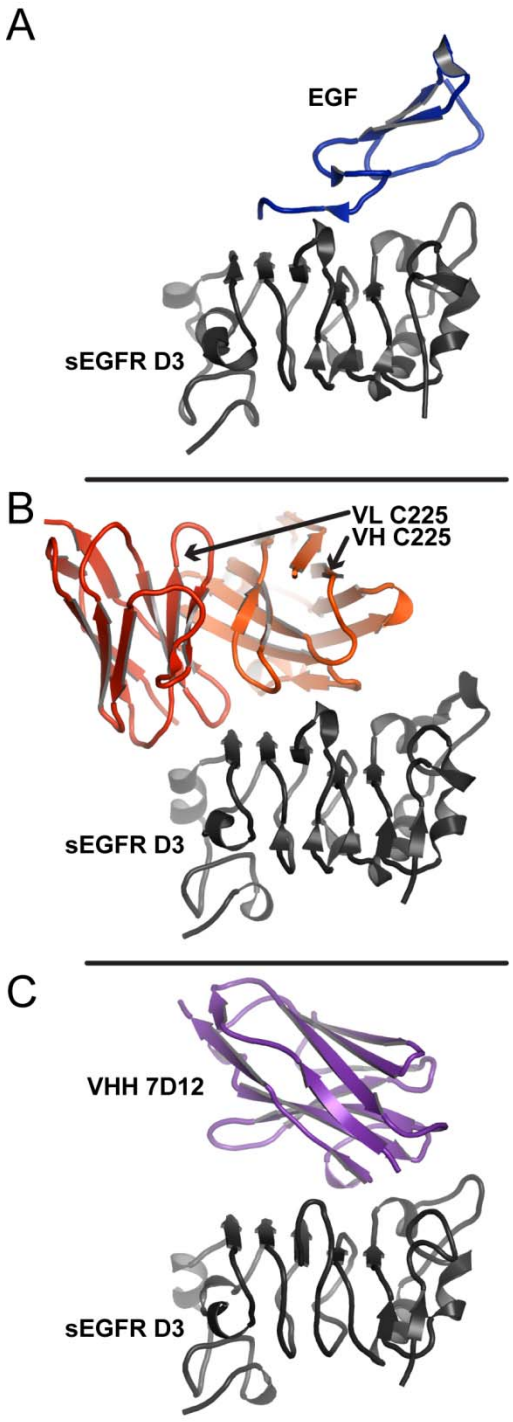

Figure 8. VHH 7D12 binds the ligand binding surface of EGFR domain 3. A) Cartoon representation of EGF (blue) bound to domain 3 of EGFR (gray), from pdb 1IVO. B) Heavy (orange) and light (red) chain variable Ig domains of Fab C225 bound to domain 3 of EGFR, from pdb 1YY9. C225 partially overlaps with the ligand binding surface. C) VHH 7D12 (violet) bound to domain 3 of EGFR. 7D12 extensively overlaps the ligand binding region on domain 3.

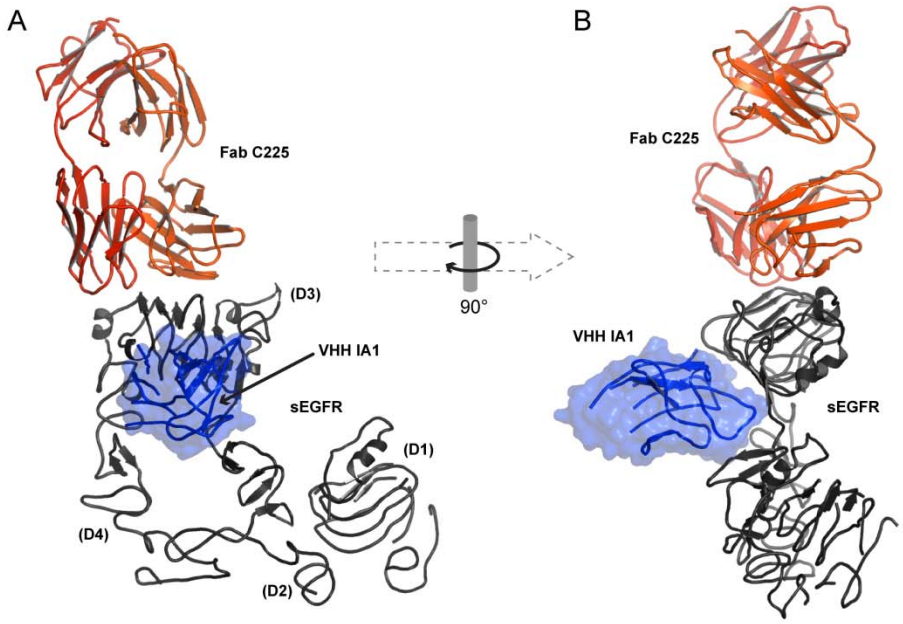

Figure 9. VHH IA1 binds to a novel epitope on the sEGFR domain 3. A) Cartoon representation of the crystal structure of a complex composed of VHH IA1 (blue), Fab C225 (heavy chain – orange; light chain - red), and sEGFR (gray). B) Complex viewed after an approximate 90 clockwise rotation around a vertical axis. Fab C225, domain 3, and the paratope region of VHH IA1 are well ordered in the structure, while domain 1 and portions of domain 2 and 4 are significantly disordered. A molecular surface corresponding to full VHH IA1, based on the crystal structure of unbound IA1, is shown. The VHH IA1 epitope is distinct from the Fab 225 epitope and the ligand binding site.